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Papillomavirus Virus-like Particles for the Delivery of Multiple Cytotoxic T Cell Epitopes

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Chimeric papillomavirus (PV) virus-like particles (VLPs) based on the bovine papillomavirus type 1 (BPV-1) L1 protein were constructed by replacing the 23-carboxyl-terminal amino acids of the BPV1 major protein L1 with an artificial "polytope" minigene, containing known CTL epitopes of human PV16 E7 protein, HIV IIIB gp120 P18, Nef, and reverse transcriptase (RT) proteins, and an HPV16 E7 linear B epitope. The CTL epitopes were restricted by three different MHC class I alleles (H-2^b, H-2^d, HLA-A*0201). The chimeric L1 protein assembled into VLPs when expressed in SF-9 cells by recombinant baculovirus. After immunization of mice with polytope VLPs in the absence of adjuvant, serum antibodies were detected which reacted with both polytope VLPs and wild-type BPV1L1 VLPs, in addition to the HPV16E7 linear B cell epitope. CTL precursors specific for the HPV16 E7, HIV P18, and RT CTL epitopes were also detected in the spleen of immunized mice. Polytope VLPs can thus deliver multiple B and T epitopes as immunogens to the MHC class I and class II pathways, extending the utility of VLPs as self-adjuvanting immunogen delivery systems.

INTRODUCTION

Cytotoxic T lymphocytes (CTL) play a key role in eradication of some infectious diseases and cancer by the immune system (Byrne *et al.,* 1984; McMichael *et al.,* 1983; Feltkamp *et al.,* 1995). Novel vaccines capable of inducing protective CTL responses have used recombinant protein expressed in the experimental animals using viral (Perkus *et al.,* 1995) or bacterial vectors (Shen *et al.,* 1995) or nucleic acids (Yang *et al.,* 1995). Induction of a broad response directed simultaneously against multiple CTL epitopes spread over multiple antigenic proteins may be necessary for the development of successful vaccines against many viral diseases, where immune selection pressure may produce viral escape mutants. An alternative strategy for CTL induction has been the use of mixtures of synthetic peptide epitopes formulated in appropriate adjuvants (Scalzo *et al.,* 1995; Vitiello *et al.,* 1995). To enable the induction of multiple immune responses, this approach has been extended to include various combinations of epitopes synthesized or expressed as a single defined nucleic acid or protein (An and Whitton *et al.,* 1997; Thomson *et al.,* 1996, 1998; Rodriguez *et al.,* 1998; Hanke *et al.,* 1998; Mateo *et al.,* 1999; Woodberry *et al.,* 1999). However, such constructs still require experimental adjuvants for the successful induction of immune responses, and the ability of these adjuvants to induce sustained CTL responses is not yet established.

Papillomavirus (PV) major capsid protein L1, alone or with the minor capsid protein L2, can self-assemble into virus-like particles (VLPs) when expressed in eukaryotic and prokaryotic systems (Zhou *et al.,* 1991b; Roden *et al.,* 1996). These VLPs are highly immunogenic with or without adjuvant and elicit high titers of systemic neutralizing antibodies, which provide protection from experimental challenge with infectious virus in animal papillomavirus models (Christensen *et al.,* 1996; Kirnbauer *et al.,* 1996; Lowe *et al.,* 1997). C-terminal truncation mutants of PV L1 protein also form VLPs (Paintsil *et al.,* 1996), and up to 60 amino acids can be added to this region of L1 without disrupting the assembly of VLPs (Müller *et al.,* 1997). Thus, when an H-2D^b restricted HPV16 E7 CTL epitope (aa318–327) (Feltkamp *et al.,* 1993) or an H-2D^d restricted HIV P18 CLT epitope was added to the C-terminus of bovine papillomavirus type 1 (BPV1) L1, the recombinant of BPV1 L1 proteins self-assembled into VLPs which elicit immunity to PV and also to the incorporated CTL epitopes (Peng *et al.,* 1998).

We therefore made a chimeric BPV-1 L1 protein incorporating multiple foreign B and T cell epitopes to investigate whether it was possible to use the self-adjuvanting properties of the papillomavirus VLP delivery system to induce multiple immune responses using a single defined particle immunogen.

RESULTS

Production of chimeric BPV1L1 polytope VLPs

A recombinant baculovirus expression system was chosen to express a BPV1L1 polytope fusion protein. This contained three HIV IIIB and one HPV CTL epitopes in addition to a single HPV16 E7 antibody epitope in a

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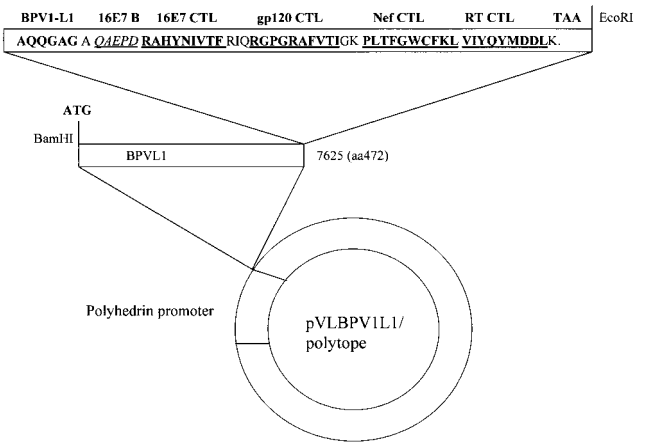

FIG. 1. Construction of PVLBPV1L1 polytope transfer vector. The BPV L1 gene, truncated after the codon for residue 472, was extended by sequential PCR with overlapping primers to include DNA encoding the entire polytope sequence as shown. This chimeric L1 construct, flanked by *Bam*HI and *Eco*RI sites, was cloned into a baculovirus intermediate vector pVL1393 at these sites.

48-amino-acid sequence (Table 1) that was substituted for the 23 C-terminal amino acids of the natural BPV-1 L1 sequence (Fig. 1). Deletion of the 23 most C-terminal amino acids of BPV1 L1 does not interfere with L1 self-assembly into VLPs (Paintsil *et al.,* 1996), and a hybrid HPV16 L1 fusion protein in which 60 foreign amino acids are substituted for the C-terminal 23 amino acids of HPV16 L1 can form VLPs (Müller *et al.,* 1997). The BPV1 L1 polytope protein was expressed in Sf9 cells using recombinant baculovirus, and VLPs were purified from cell lysate by density gradient in the usual manner. Fractions of the gradient were subjected to SDS–PAGE and a band of around 60-kDa size was visible by Coomassie blue staining of a fraction of density 1.30 g/ml. Compared with wild-type BPV1L1, the apparent molecular weight of the hybrid BPV1L1 was higher, as expected. The identity of this band as an L1 fusion protein was confirmed by immunoblot using L1-specific monoclonal antibodies (Fig. 2). Electron microscope analysis of the gradient fraction containing L1 showed virus-like particles of heterogeneous size, with some loss of icosahedral structure when compared with the wild-type BPV1L1 VLPs (Fig. 3). The BPV-1 L1 polytope protein formed VLPs from L1 protein with three- to fivefold lower efficiency, when compared with the wild-type BPV1L1 as judged by the yield of L1 protein in VLPs when compared with total L1 protein expressed in rBV-infected cells (data not shown).

Antibody response to polytope VLPs

To test the ability of polytope BPV1L1 VLPs to induce anti-BPV1L1 antibody, groups of BALB/c, C57BL/6, and HLA-A2.1K^b transgenic mice were immunized twice with 20 μg BPV1L1 polytope VLPs. Anti-BPVL1 VLP antibodies were measured by capture ELISA, which is relatively specific for conformational antibody to VLPs. All immunized mice produced antibodies that recognized both polytope and native BPV1L1 VLPs. The titer of antibody recognizing wt BPV1L1 VLPs was lower than that against polytope VLPs (Fig. 4). In contrast, antiserum raised using WT-BPV1L1 VLPs recognized BPV1L1 VLPs better than polytope VLPs (Fig. 4). These data suggest that the dominant B epitopes of the polytope BPV1L1 VLPs were different from those of the WT BPV1L1 VLPs. This observation, taken together with the EM appearance of the particles and the immunogenicity data, suggests that foreign antigens fused to the BPV1L1 C-terminus could have a significant impact on the integrity of the L1 VLPs.

Sera from mice immunized with polytope VLPs were also tested in ELISA using the incorporated E7 B epitope as substrate. Peptide-specific antibody was measured in both C57BL/6 and BALB/c mice, and only very low E7 antibody was measured in HLA-A2.1/K^b transgenic mice. The titer of E7 peptide-specific antibody was quite low compared to that of specific anti-L1 antibody (Fig. 5) suggesting that the E7 B cell epitope, which is a strong epitope when E7 is the immunogen, is subdominant to conformational VLP B epitopes when contained in a chimeric VLP.

In vivo induction of CTL responses by polytope VLPs

Polytope BPV1L1 VLPs carried four CTL epitopes restricted by three different MHC alleles (Table 1). To evaluate the ability of the polytope VLPs to prime CTL responses *in vivo* to each of the CTL epitopes, C57BL/6 (H-2^b) mice, BALB/C (H-2K^d), and HLA-A2.1.K^b transgenic mice were immunized with purified BPV1L1 polytope VLPs in PBS, with PBS only, or with denatured VLPs as control. Splenocytes harvested 6 days after the second immunization were restimulated with synthetic peptides

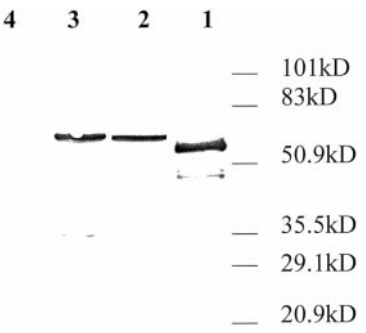

FIG. 2. Immunoblot detection of polytope VLPs protein. CsCl gradient-purified wild-type BPV1L1 VLPs, polytope VLPs, and wild-type baculovirus were separated by SDS–PAGE, blotted onto nitrocellulose membrane and probed with MAb MC15, and reactivity was detected by enhanced chemiluminescence. Protein markers are shown on the right. Lane 1, BPV1L1 VLPs. Lanes 2 and 3, polytope VLPs. Lane 4, wild-type baculovirus.

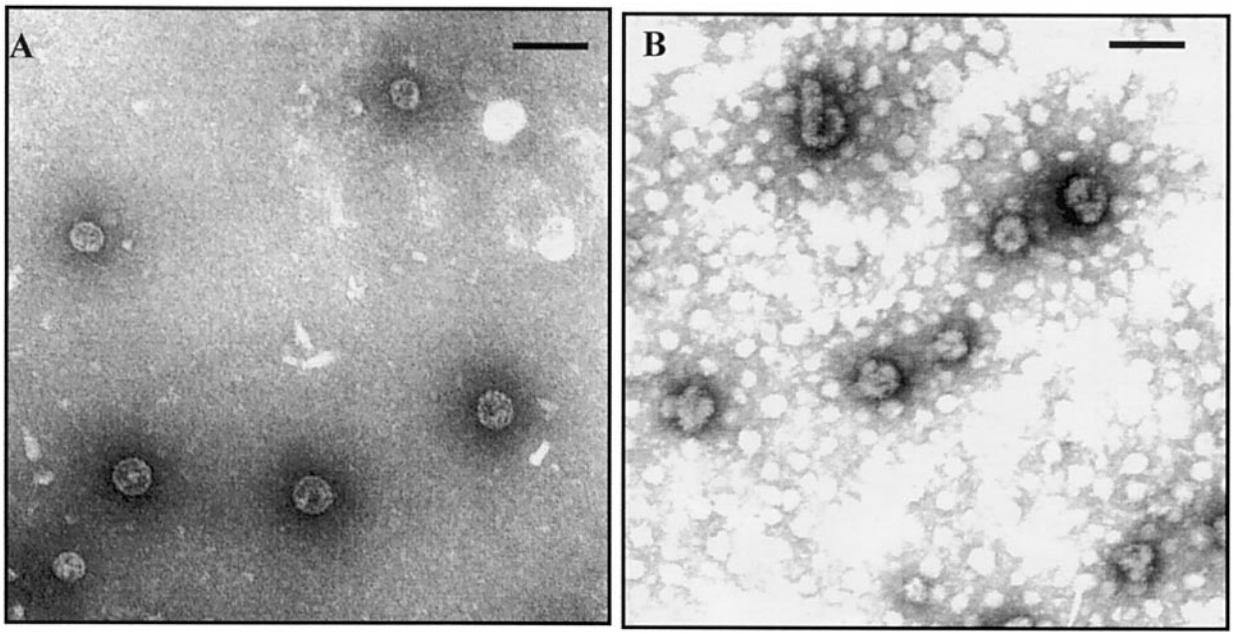

FIG. 3. Electron microscopy of purified wild-type BPV1L1 VLPs. (A) BPV1L1 VLPs. (B) Hybrid BPV1L1 polytope VLPs. The bar represents 100 nm in each case.

corresponding to the recall epitopes *in vitro* to generate bulk effector CTL, which were then used in standard chromium-release assays against peptide-sensitized or transfected tumor targets. The bulk effector CTL from the C57BL/6 mice specifically lysed HPV16 E7 peptide-coated EL4 and E7 transfected tumor target cells (C2) (Fig. 6), but did not lyse EL-4 cells in the absence of E7 peptide, confirming the specificity of the CTL responses. The bulk effector CTL from the BALB/c mice similarly lysed HIV P18 peptide-coated P815 cells. The bulk effector CTL from HLA-A2.1/K^b transgenic mice recognized HIV RT-polymerase peptide-coated EL4S3-HHD cells, but did not recognize Nef peptide-coated target cells. For this peptide immunogen, the peptide sequence used in the CTL assay differed by one amino acid residue from that incorporated in the polytope construct (Table 1). To confirm that the immunogenicity of the polytope VLPs was dependent on their VLP conformation, mice were immunized with native configuration, denatured polytope VLPs, or PBS, and no CTL response was observed in mice so immunized (data not shown).

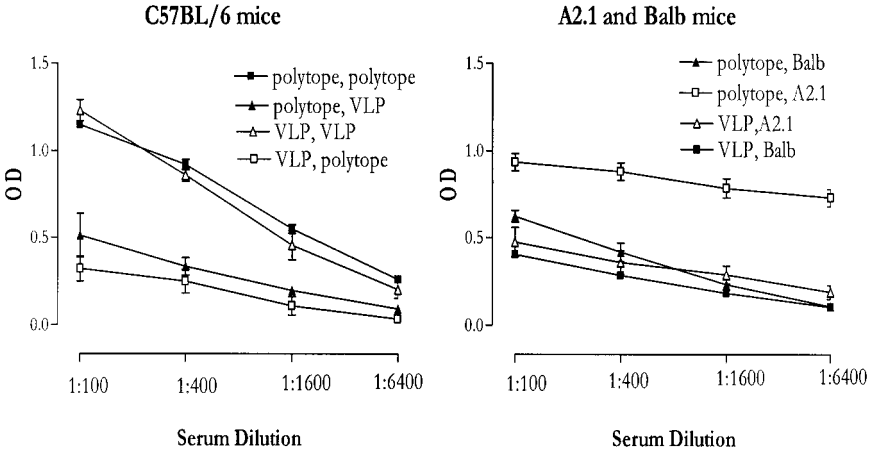

FIG. 4. Antibody responses induced in three mouse strains by immunization with BPV1L1 polytope VLPs without adjuvant (Poly VLP) or by BPV-1 VLPs without adjuvant (VLP). Left: Specific antibody induced by polytope VLPs (filled symbols) and VLPs (open symbols) in C57Bl/6 mice was detected by a capture ELISA against intact BPV1L1 polytope VLPs (squares) or wild-type BPV1L1 VLPs (triangles). Right: specific antibody induced by polytope VLPs against polytope VLPs (squares) or VLPs (triangles) is shown for Balb/c (open symbols) or A2.1/Kb transgenic mice (filled symbols). Each point represents data from pooled sera from a group of five mice.

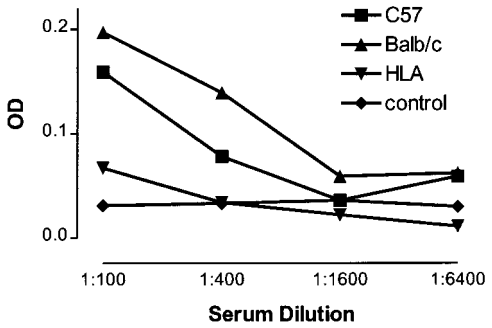

FIG. 5. Antibody responses against E7 peptide induced in three mouse strains by immunization with BPV1 L1 polytope VLPs without adjuvant. Specific antibody was detected by a peptide ELISA against E7 antibody epitope. Negative control is sera from mice immunized with PBS only. Each point represents data from pooled sera from a group of five mice.

DISCUSSION

VLPs are candidate antigen delivery systems for the induction of neutralizing antibodies and of CTL epitopes through the MHC class I presentation pathway. The current results demonstrate the feasibility of delivering multiple CTL epitopes in a single VLP construct to induce responses against many epitopes across individuals with diverse MHC backgrounds.

CTL responses have been shown to play a critical role in the elimination of virus infection or clearance of tumor, so that an effective vaccine against viral infection might require incorporation of many different CTL specificities to ensure protection against multiple viruses on a heterogeneous collection of MHC backgrounds. CTL epitope-based plasmids encoding MHC class I, class II, and antibody-binding epitopes, delivered by immunizing with recombinant DNA or by infecting with recombinant viruses, have been shown to be immunogenic in mice and to confer protection against viral infection and cancer (An and Whitton, 1997; Gilbert *et al.,* 1997; Thomson *et al.,* 1996, 1998; Rodriguez *et al.,* 1998; Hanke *et al.,* 1998; Suhrbier, 1997), but hazards related to the nature of the delivery systems may limit their usefulness as human vaccines. Peptides, comprising multiple CTL epitopes conjoined in a single, artificial construct, have been formulated in various adjuvants which target the MHC class I pathway and lead to the generation of CTL responses (Vitiello *et al.,* 1995). VLPs can mimic the immunological function of a virus, but are not infectious and, consequently, have been trialled as vaccines. Recombinant Ty-VLPs carry a string of up 15 defined CTL epitopes from plasmodium species prime protective CTL responses in mice following a single administration without adjuvant

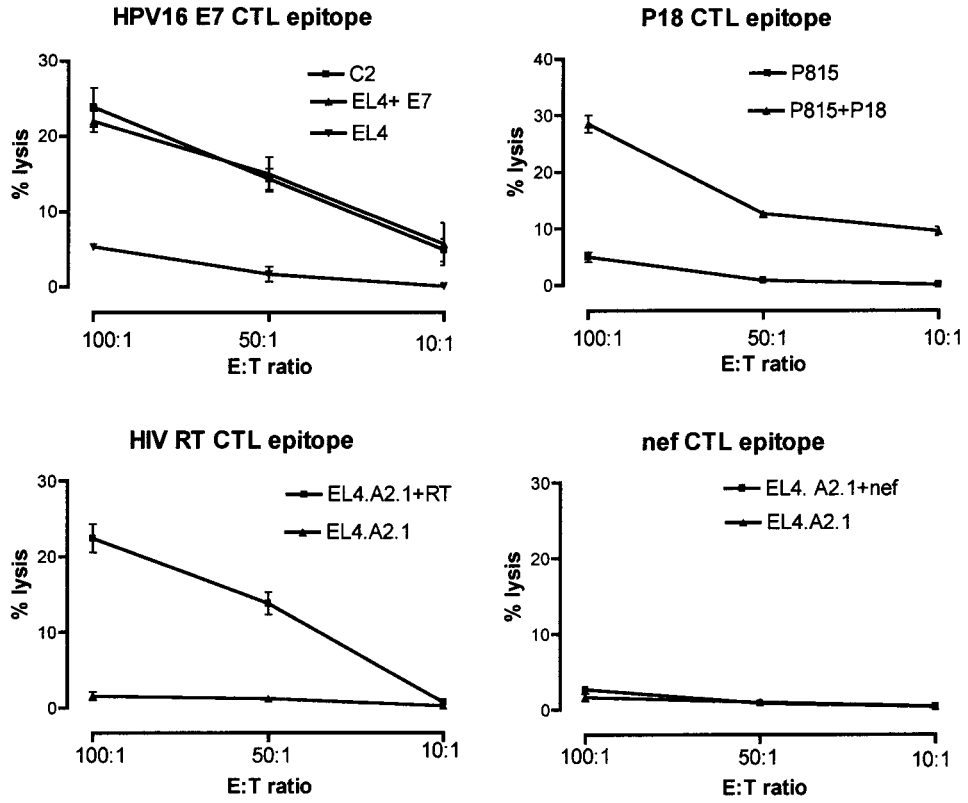

FIG. 6. Lysis of target cells presenting four different epitopes by splenocytes from murine BPV1L1 polytope VLP vaccinated mice. Splenocytes from BALB/c (H-2D^d), C57BL/6 (H-2D^b), and HLA-A.21K^b transgenic mice, immunized with polytope VLPs, were stimulated *in vitro* with the appropriate peptide. The generated effector cells were used in standard ^{51}Cr-release assays against peptide-coated or uncoated target cells and, for E7, additionally against the C2 cell line expressing endogenous E7.

(Gilbert *et al.,* 1997). VLPs apparently possess an inherent adjuvant activity which allows for effective MHC class I-restricted presentation of CTL epitopes. This is likely due their particulate nature and to the expression of α6 integrin, the putative PV receptor (Evander *et al.,* 1997), on dendritic cells (Al Jabbir, submitted).

Several antigen delivery systems based on VLPs induce CTL activity. Hybrid HIV gag particles induce a strong anti-V3 loop CTL response (Griffiths *et al.,* 1993). Chimeric hepatitis B surface antigen particles containing HIV-1 determinants or HPV16 E7 determinants stimulate specific CTL responses (Michel *et al.,* 1993; Tindle *et al.,* 1994). Recombinant parvovirus-like particles harboring a CTL epitope from lymphocytic virus (LCMV) nucleoprotein induce complete protection of mice against a normally lethal LCMV infection (Sedlik *et al.,* 1997). PV VLPs are highly immunogenic when administered to mice, primates, and humans (Zhang *et al.,* 2000; Nardelli-Haefliger *et al.,* 1999; Lowe *et al.,* 1997), suggesting that chimeric VLPs might act as effective "dual-purpose" vaccines for inducing neutralizing antibodies which prevent infection and CTLs that eradicate established infections. The papillomavirus major protein, L1, has a carboxyl-terminus that encodes a nuclear localization signal but otherwise shows relatively low sequence homology between different PV types (Zhou *et al.,* 1991a). Deletion of part of the BPV1L1 C-terminal sequence does not interfere with L1 self-assembly into VLPs and, in contrast, the yield is increased by an unknown mechanism (Paintsil *et al.,* 1996). Efficient formation of VLPs was obtained with a recombinant construct in which the carboxy-terminal 34 amino acids of HVP16 L1 were replaced by up to 60 amino acids, and this modification did not interfere with basic structural and functional properties of the particles (Müller *et al.,* 1997). The carboxyl terminus 23 amino acids of bovine papillomavirus type-1 L1 protein is not required for capsid formation, and it can be replaced by at least some inserted sequence without decreased VLP formation. Bovine papillomavirus L1 VLPs containing defined CTL epitopes including HIV P18 CTL epitope or HPV16 E7 CTL epitope were able to induce CTL responses through the MHC class I pathway (Peng *et al.,* 1998), and the resulting response could inhibit growth of an E7 tumor cell line *in vivo.* Chimeric human papillomavirus type 16 L1 VLPs containing the first 60 amino acids of HPV16 E7 protein could induce E7-specific CTLs and protected mice from tumor growth and induced regression of tumors *in vivo* (Schäffer *et al.,* 1999). HPV16 L1 VLPs incorporating an HPV16 L2- E7 fusion protein could protect immunized mice from challenge with a tumor-expressing cell line expressing E7, although E7-specific CTL were not demonstrated (Greenstone *et al.,* 1998). All of these data indicated that chimeric papillomavirus VLPs are useful delivery systems for tumor immunotherapy. Given that up to 60 amino acids can be fused to the carboxy-terminus of L1 without affecting VLP assembly, we wished to determine whether we could incorporate more than one CTL epitope into the chimeric particle to circumvent the problem of MHC polymorphism in outbred populations. We hypothesized that these particles would be able to induce a CTL response against each added epitope in addition to the L1 portion. In our study, three of four CTL epitopes in the polytope VLP constructs were clearly capable of inducing a MHC-I-restricted CTL response in the appropriate mouse strain in the absence of adjuvants. In our system, replacement of 23 amino acids of the BPV1L1 C-terminus with a minigene encoding 48 amino acids markedly decreased the formation of VLPs and caused significant morphology changes when compared with the wild-type BPVL1 VLPs. In contrast, insertion of the entire GFP gene (encoding 237 amino acids) to the C-terminus of BPV1L1 permitted the formation of normal size and fluorescent VLPs, but the yield was extremely reduced (our unpublished observations), whereas 30 amino acids of the influenza matrix protein, when fused to the C-terminus of HPV16 L1, produced only small numbers of intact VLPs (Müller *et al.,* 1997). Modifications the C-terminus of L1 thus do not always result in stable PV VLP formation, and stability depends not only on the length of insert but also on the nature of the inserted gene.

Chimeric VLPs have been demonstrated in a hemagglutination assay (Roden *et al.,* 1995) to induce antibodies to conformational epitopes of PV, as are required for virus neutralization in CRPV challenge systems, after immunization with or without adjuvant (Müller *et al.,* 1997; Liu *et al.,* 1998; Peng *et al.,* 1998). Several studies have shown that VLPs and chimeric VLPs can bind to and penetrate cells in a manner similar to that of infectious virions (Müller *et al.,* 1997; Muller *et al.,* 1995; Zhou *et al.,* 1995) and suggest that modification of the native VLP by the insertion of a foreign gene does not interfere with this function of VLPs. However, in our study, the serum from all three strains of mice immunized with polytope chimeric VLPs reacted with polytope VLPs better than WT-type BPV1L1 VLPs. In contrast, the serum from wild-type BPV1L1 immunized mice gave greater reactivity against BPV1L1 VLPs than against polytope VLPs. Together with morphological data, this observation allows the conclusion that structural and functional properties of our polytope VLPs are altered by the inclusion of a foreign gene at the C-terminus.

This study also addressed whether CTL epitopes might be delivered together with sequences containing B cell epitopes other than L1. Although the HPV16 E7 B cell epitope included in the chimeric VLPs was highly immunogenic as a peptide coupled with GST (Fernando *et al.,* 1998), a range of mice immunized with polytope VLPs produced only low levels of E7 antibody to this epitope. Furthermore, chimeric VLPs did not react with E7-specific antibodies when examined by capture ELISA (data not shown). These observations are in agreement with

the proposed partial or complete internal localization of the inserted sequences within the chimeric VLPs (Müller *et al.,* 1997).

Both HPV and HIV are sexually transmitted viral diseases and are currently the focus of numerous vaccine studies. The present study has coupled the papillomavirus L1 VLPs with HPV16 E7, HIV gp160, RT-polymerase, and nef protein CTL epitopes. The strong CTL response against HPV16 E7, HIV P18, and RT-polymerase induced by polytope BPV1L1 VLPs suggests that it is possible to design a VLP-based vaccine against more than one sexually transmitted virus. The present study has demonstrated that hybrid PV VLPs can be used as an efficient antigen delivery system to deliver more than one CTL epitope through MHC class I pathways, although we have not tested for two discrete CTL responses in the same animal. The length and site of optimal foreign protein incorporated into the L1 VLPs and the stability of the resulting chimeric VLPs may, however, require empiric definition if polytope VLPs are to be considered an immunization strategy for controlling several viral infections on several MHC backgrounds using a single immunization.

MATERIALS AND METHODS

Mice, cell lines, and peptides

Adult female C57BL/6J, BALB/c, and HLA-A2.1/K^b transgenic (H-2^b) mice were purchased from the Animal Resource Center (Australia). The HPV 16 E7 transfected EL4 cell line C2 (Tindle *et al.,* 1995), the parent line EL4, and the P815 cell line were maintained in complete RPMI 1640 medium plus 10% fetal bovine serum (FBS, CSL, Australia). The HLA-A2.1/K^b transfected EL4 cell line, EL4S3-HHD, was obtained from Dr. Andreas Suhrbier and was maintained in complete RPMI medium containing 10% FBS plus 400 μg/ml G418. Peptides synthesized using F-moc chemistry were purchased from Chiron (Melbourne, Australia).

Construction of recombinant baculovirus transfer vectors

The following three primers, designed to fuse the polytope gene to the C-terminus of BPV1L1 sequence, deleting nucleotides from 7625 downstream, were purchased from Life Technology.

Primer A: 5′-ATTGTAACAAATGCTCTCCCTGGTCCTCTCTGGATACGAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTGCTTGTGCTCCTGCCCCTTGCTGTGCTA-3′;

Primer B: 5′-CGGAATTCTTATTTCAAATCATCCATGTATTGGTAGATAACTAGCTTGAAGCACCACCCAAAGGTCAGGGGTTTTCCTATTGTAACAAATGCTCTCCC-3′;

Primer C: 5′-CCGGGATCCATGGCGTTGTGGCAACAAGGCCAGAAGC-3′.

TABLE 1

Epitopes Included in the Polytope VLPs

Epitope description	Amino acid sequence	Restriction
HPV16E7 B epitopes	QAEPD	
HPV16E7 CTL epitope	RAHYNIVTF	H-2 D^b
HIV gp120 P18 CTL epitope	RIQRGPGRAFVTIGK	H-2 D^d
HIV RT CTL epitope	VIYQYMDDL	HLA-A2.1
HIV Nef CTL epitope	PLTFGWCYKL[a]	HLA-A2.1

[a] The predicted sequence encoded by the polytope PLTFGWCFKL differs at residue 8 from the naturally occurring epitope used as target for the CTL responses.

Primer A and primer B represent a 144-bp insert sequence. The nucleic acid sequence of the fusion coded for one flanking *Eco*RI restriction site, a stop codon, and four CTL epitopes restricted by three MHC alleles and a B cell epitope from HPV16 E7 (Table 1). The primer C coded for one flanking *Bam*HI restriction site. Initially primer A, primer C, and the BPV1L1 gene were used to fuse DNA encoding the first 27 amino acids of the polytope construct to BPV1L1. Similarly, primer B and primer C and the first PCR product were used to extend this intermediate construct to form the complete hybrid BPV1L1 gene. The PCR products were digested with *Bam*HI and *Eco*RI and inserted into a baculovirus transfer vector, pVL1393 (Pharmingen), at the *Bam*HI and *Eco*RI sites. The ligation product was used to transform *Escherichia coli* DH α-5 cells. The correct recombinant clones were confirmed by the existence of *Bam*HI and *Eco*RI sites and also sequenced by dideoxynucleotide procedures to determine the orientation and integrity of the inserted sequences.

Construction of recombinant baculovirus

Spodoptera frugiperda (Sf9) cells were grown at 28°C as suspension or monolayer cultures in TNMFH medium (Sigma) supplemented with 10% FBS, 2 mM glutamine. For recombinant baculovirus construction, 10 μg plasmid was used to transfect Sf9 cells together with 2 μg of linearized Baculo-Gold DNA (Pharmingen, San Diego, CA). Recombinant viruses were purified by standard methods as suggested by the manufacturer. To test for the expression of BPV1L1 capsid protein, 10^6 Sf9 cells were infected with baculovirus recombinants at an m.o.i. of 5 to 10. After incubation, medium was removed and the cells were washed with PBS. The cells were then lysed in SDS–sample buffer and analyzed by SDS–PAGE and immune blotting assay.

Production and purification of VLPs

The production and purification procedures for VLPs have previously been described (Qi *et al.,* 1996). In general, Sf-9 insect cells were infected with recombinant

baculovirus at an m.o.i. of 10 per cell and incubated at 28°C for 72 h. The cells were centrifuged, washed once with PBS, and resuspended in an appropriate amount of PBS in the presence of 2 mM PMSF. The cell suspension was homogenized with a Dounce homogenizer and then centrifuged at 3000 rpm for 10 min at 4°C to separate the nuclear fraction. The pellet was resuspended in an appropriate resuspension buffer and sonicated for 45 s on ice. The nucleus suspension was then loaded onto a 20% sucrose cushion and centrifuged at 26,000 rpm in a Beckman SW-26 rotor at 4°C for 2 h. The pellets were resuspended with resuspension buffer and sonicated again for another 45 s. This resuspension was then mixed with CsCl and centrifuged at 20°C with a Beckman SW-41 rotor for 20 h. A band with a density of 1.28 g/ml was collected and dialyzed extensively against PBS.

Immunoblot analysis

Protein samples were diluted in SDS–PAGE sample buffer, boiled at 100°C for 10 min, and electrophoresed through a 10% SDS–PAGE gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS and probed with the anti-L1 monoclonal antibody MC15 (Kulski *et al.,* 1998) at a dilution of 1:2000. Bound antibody was detected by incubation of the membrane with horseradish-peroxidase-conjugated sheep anti-mouse antibody (Silenus, Australia) at a dilution of 1:1000 and visualized using enhanced chemiluminescence (Amersham).

Transmission electron microscopy

Samples of the VLP fractions from the CsCl gradients were dialyzed against 10 mM HEPES (pH 7.5) for 45 min on floating filter pads (0.02-μm pore size, Millipore). Carbon-coated copper grids (200-mesh size; EM Sciences) were treated with 20 μl of poly-L-lysine (1 mg/ml; Sigma) for 2 min. The sample was placed onto the grid for 2 min. Spotted grids were then stained with 30 μl of a 2% uranyl acetate solution for 2 min. Excess stain was removed and grids were air dried. Specimens were then examined with a Zeiss EM 900 electron microscope at 80 kV.

Cytotoxic T lymphocyte assays

Eight to 10-week-old female BALB/c, C57BL/6, and HLA-A2.1K^b transgenic mice were immunized by intramuscular injection with 20 μg of polytope BPV1L1 VLPs in PBS and subsequently boosted at day 21. Control mice were immunized with PBS, polytope VLPs denatured by boiling at 100°C for 10 min, or wild-type VLPs. Mice were sacrificed on day 28, spleens were removed, and a single cell suspension was held in complete RPMI 1640 medium supplemented with 10 U/ml rIL-2 (Sigma) and stimulated with peptide for 3 days. For HIV p18 CTL, the effectors were generated by restimulation with HIV p18 peptide RIQRGPGRAFVTIGK (single letter AA code). For HPV16 E7 CTL, effectors were generated by restimulation with HPV16 E7 CTL peptide RAHYNIVTF. For HIV RT-polymerase, effectors were generated by restimulation with HIV RT-polymerase peptide VIYQYMDDL. For HIV *nef* CTL, effectors were generated by restimulation with HIV *nef* peptide PLTFGWCYKL. The predicted amino acid sequence of the corresponding region of the polytope is PLTFGWC**F**KL. The effectors were then used in standard ^{51}Cr-release assays against peptide-sensitized targets. Peptide-sensitized target cells were sensitized with 2 μg/ml peptide for 2 h at 37°C followed by two washes in medium. Effector cells and labeled target cells were plated into 96-well round-bottom plates at various effector/target ratios and incubated at 37°C, 5% CO_2, for 5 h. A total of 25 μl of supernatant was collected to Lumaplates from each well, dried at 42°C, and counted with a gamma counter. The percentage of specific lysis was calculated as specific lysis = (sample release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum release was generated by adding 100 μl of 10% SDS to 100 μl of target cells, and spontaneous release was assayed from 100 μl of target cells incubated with 100 μl of medium. Assays were performed in triplicate, and spontaneous ^{51}Cr release from the various targets did not exceed 15%.

ELISA assays for anti-VLP serum IgG

Measurement of total VLP-specific IgG in serum was performed in flat-bottom microtiter plates, as previously described (Peng *et al.,* 1998). Briefly, a rabbit anti-BPV1 antiserum was used to coat the plates. After blocking with 5% milk/PBS, 50 μl VLPs at a concentration of 10 μg/ml was added to each well and incubated at 37°C for 90 min. Sera were tested by fourfold serial dilution in 5% milk/PBS. Bound antibody was detected with horseradish-peroxidase-conjugated sheep anti-mouse IgG at a dilution of 1:1000, followed by incubation with OPD in the presence of hydrogen peroxide. The reaction was stopped with 3 N HCl and absorbance was measured at 450 nm.

Peptide ELISA

Microtiter plates (Dynatech) were coated overnight with 50 μl of E7 antibody peptide (Fernando *et al.,* 1998). After being blocked for 2 h at 37°C with 100 μl of 5% BSA, 0.05% Tween 20, in PBS, plates were washed three times with PBS/0.05% Tween 20. A total of 50 μl of sera at various dilutions was added for 1 h. Plates were washed again and 50 μl of goat-anti-mouse IgG peroxidase conjugate was added at a 1:5000 dilution. After 1 h, plates were washed before the addition of ABTS substrate (0.2 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M Na–acetate–phosphate buffer [pH 4.2] with 4 μl of 30% H_2O_2 per 10 ml). Absorbance was measured

after 1 h at 490 nm in a Dynatech automated plate reader. As a control, readings from wells coated with peptide were compared to wells coated with PBS only.

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